REMARKS

I. Status of the Claims

Claims 1, 2, 5-11, and 13 are pending. Claims 1, 2, 5-11, and 13 are rejected.

II. Diagrams from Applicants' Interview Dated September 8, 2009

Both Lee and Studer, the primary references used to reject the claims, disclose a five-step method. Lee's methods provided dopaminergic neurons and some serotonergic neurons (using the same protocol).

Studer disclosed specific modifications of Lee's five-step protocol for the purpose of producing other types of neural-related cells, namely, oligodendrocytes, astrocytes, GABA-ergic neurons, and serotonergic neurons. Except for one suggested modification (for dopaminergic neurons, where SHH and FGF8 are added at Stages I and II), the first three stages do not involve incubating cells with any of the claimed factors. The factors are added at Stage IV. Further, the only protocol in which all four claimed factors are present is for producing dopaminergic neurons. In this scenario, there is a list of sixteen factors that might be added at Stage IV and/or Stage V. BDNF is one.

III. The Rejections

A. Rejection Under 35 U.S.C. § 103: Studer/Lee

Claims 1, 2, 5-9, 11, and 13 remain rejected under 35 U.S.C. § 103(a) on the grounds that they are unpatentable over WO 02/086073 (Studer) and U.S. 2003/0211605 (Lee) for the reasons of record. Applicants respectfully traverse the rejection.

In Applicants' previous response, Applicants explained how each factor induces a specific phenotypic effect so that, if factors are added simultaneously, it is not reasonably predictable that the phenotype will be the same as the phenotype produced by exposing the cell to the factors sequentially. This argument was supported by the Declaration of Dr. Catherine Verfaillie, an internationally-known stem cell scientist

and expert in the field. Nevertheless, the Examiner maintains the rejection for the reasons of record and erroneously dismisses the Declaration of Dr. Verfaillie.

First, on page 5 of the Office Action, the Examiner states "In contrast to Applicants' arguments, the Examiner asserts that the combined references do render the claimed invention obvious because the claimed method uses the same growth factors and same ES cells."

At the outset, Applicants point out that claim 8 is not directed to the "same cells." The cells of claim 8 are not embryonic stem cells. Clarification is requested.

The above point notwithstanding, the Examiner again makes the argument that the same result would be obtained because the same growth factors are used. The Examiner does not address the issue of the expected effect of sequential exposure. Moreover, the Examiner provides no scientific evidence to rebut the statements of Dr. Verfaillie regarding the differences of these approaches.

The Examiner dismisses Dr. Verfaillie's Declaration on the grounds that Dr. Verfaillie "fails to provide side-by-side comparisons to demonstrate that the claimed cell types, or end products generated from sequential addition of growth factors, are different from those that are simultaneously exposed to the same growth factors taught in the cited references."

This is an erroneous reason to dismiss this Declaration. Obviousness is premised on: (1) what the person of ordinary skill would have been motivated to do; and (2) whether the person of ordinary skill would have reasonably expected that they would successfully produce the results. Thus, the opinion of Dr. Verfaillie goes to what the person of ordinary skill in the art would have expected and not to what actually occurred after the fact. A side-by-side comparison shows what actually occurs. But the proper question for obviousness is what the person of ordinary skill would have expected.

Dr. Verfaillie explains that the person of ordinary skill would have expected different results from simultaneous and sequential exposure, even to the same growth factors. Thus, having modified the

procedure of the cited references as the Examiner suggests, the person of ordinary skill in the art would not have known what to expect. They would not have reasonably expected that they would successfully produce the end product (for example, dopaminergic and serotonergic neurons) as were produced by the prior art method. Thus, they might have been motivated to try some modification or other, but, again, they would not have been able to reasonably expect a successful end result.

Furthermore, there are numerous modifications that the person of ordinary skill might have tried. But there is no guidance in either reference (or combination of references) beyond the several suggested modifications. And none of these contains the specific factors, sequence, and duration as claimed.

On pages 13-18 of this Response, Applicants present all of the exemplified and suggested protocols in the cited references and show that neither reference, alone or in combination, suggested a method with the particular claimed combination of factors, sequence, and duration.

The Declaration is also dismissed on the grounds that "the differentiated cells in the cited reference by Dr. Verfaillie are not relevant to the instant application because they are differentiated into different cells and, therefore, can't be compared with stem cell differentiated into neurons."

The evidence submitted by Dr. Verfaillie need not be directed to the same cells. Dr. Verfaillie's evidence illustrated the principle that exposure of a cell to the same factors, but in a different sequence, will not necessarily produce the same result. Applicants seek to establish what the person of ordinary skill in the art would have been motivated to do and what they would have reasonably expected, not what actually happens after the fact

On page 6, the Examiner additionally argues that the claimed method and the cited references are directed to the same goal, using the same materials. Specifically, the claimed method is directed to inducing neuronal differentiation "using the same growth factors (bFGF, FGF8, SHH, and BDNF) and the same ES cells...Although the claimed method alters the way of adding growth factors, and phenotypical cell types

may have different proportions during the recited culturing procedures, at the end of the steps, the end result of neuronal differentiation is expected and [sic] to generate dopaminergic neurons."

Again, there is no scientific explanation about why this would have been expected. There is only the unsupported assertion. But Dr. Verfaillie's Declaration explains why it would not have been expected. The Examiner fails to present any evidence to counter the scientific reasons and conclusions of Dr. Verfaillie.

Sinclair & Carroll is cited for the following: "The selection of a known material based on its suitability for its intended use supported a *prima facie* obviousness determination." This case is inapt because it does not go to the situation where a known material was used, but in a different way, as in the present case. *In re Aller* is cited for the following: "Where the general conditions of the claim are disclosed in the prior art, it is not inventive to discover the optimum or workable ranges by routine experimentation." This case is also inapt because the Applicants have not explored optimum or workable ranges. In fact, they have not explored ranges at all; they have altered how prior art components are used to create a new process. *In re Kerkhoven, In re Crockett,* and *Ex parte Quadranti* are cited as follows: "It is *prima facie* obvious to combine two compositions each of which is taught in the prior art to be useful for the same purpose in order to form a third composition to be used for the very same purpose. The idea of combining them flows logically from their having been individually taught in the prior art." This case is inapt because the method is not directed to combining two compositions to form a third composition. The present case involves using old components in a new protocol.

As Applicants have pointed out above, the Examiner provides no scientific reasons for supporting her assertions. On page 7, the Examiner responds to Applicants' arguments that the specific claimed protocol of sequential culture is not obvious over either Studer or Lee, alone or in combination. The Examiner states that "Studer and Lee do render the claimed procedures obvious" and supports this assertion with various textual citations. These begin on page 7 and end on page 8.

On page 7 of the Office Action, the Examiner argues that it would have been obvious to sequentially add the growth factors in Studer and Lee for at least seven days as claimed because of various teachings of the Studer and Lee references. Applicants have analyzed each of the cited sections and provide their comments on the relevance of each.

Lee

9:126

Lee notes that SHH and FGF8 may be added at Stages I and II, if desired, although this is "less effective" in generating dopaminergic neurons. But even if these are added during Stages I and II, it would not render the claimed protocol obvious; in fact, it would make the claimed sequence even less obvious. In the claimed protocol, the bFGF is added before the SHH and FGF8.

9:123-124

This merely explains Stage IV, which is the stage at which the CNS precursor cells are expanded (proliferated) in the presence of the various factors.

9:124

This continues discussing the proliferation medium, indicating that the medium can be supplemented with agents to encourage differentiation into neuronal cells, including bFGF and EGF. This states that during Stage IV, bFGF or EGF could be added to the other factors. Paragraph 126, therefore, goes on to state that Studer applied FGF8 and SHH at earlier stages (Stages I and II) but found that it was less effective for generating dopaminergic neurons. It states that FGF8 and SHH could be included at these stages if desired. It is not clear whether the FGF8 and SHH are only added at Stages I and II, and then not added at Stage IV. In any event, this still would not render the claim obvious, because even if they were added at Stages I and II, that would be prior to the addition of bFGF.

9:125

Here, the reference simply indicates that, in the expansion medium (Stage IV), SHH and FGF8 should be added together for a greater effect. This, then, flows into paragraph 126, that again discusses adding SHH and FGF8 and Stages I and II. It does not clarify whether the factors are added at 1 and 2 instead of 4, or at 1 and 2 in addition to 4.

9:128

This is under the section regarding the differentiation of the expanded cells that are discussed in the previous section (123-126). This indicates that the expanded precursors are induced to form mature neuronal cells by withdrawing at least one neurologic agent, typically bFGF or EGF. This, again, is consistent with all of Applicants' arguments.

Example 5

This text is also a repetition of what Applicants have already presented. It shows differentiation at Stage V, induced by withdrawing bFGF from the medium. It also refers to combined treatment with SHH and FGF8 during Stage IV expansion. So, at Stage IV, there is at least bFGF, SHH, and FGF8.

In summary, the specific claimed sequence and duration protocol is not found or suggested in any of the above text. This is not compensated by the textual citations from the Studer reference below.

Studer

5:16-17

16 indicates that bFGF can be a mitogen for generating a serotonergic neuron. 17 specifically relates to tailoring the method to generate an astrocyte by inserting an extra step after Stage IV with a mitogen

selected from the group consisting of bFGF, EGF, and PDGF. Stage IV still involves bFGF, SHH, and FGF8.

Page 26: paragraph 78

This section relates to modifying the differentiation protocol to produce different types of cells. For astrocytes, this is done by replating Stage IV cells in bFGF plus EGF or bFGF plus CNTF; for oligodendrocyte, replating Stage IV cells in the presence of mitogens, such as bFGF, EGF, and PDGF; for GABA neurons, growing Stage IV cells in the absence of SHH and FGF8, but exposing the cells in Stage V to cAMP and BDNF or cAMP andNT4.

Page 28: paragraph 85

This discusses Stage IV. It explains that factors, such as SHH and FGF8, can be added at Stage IV and that other factors could be added also, such as EGF and CNTF for astroglia cells, and PDGF or SHH for oligodendroglial cells.

Page 29: paragraph 86

This is also part of the Stage IV description. It indicates that other factors at Stage IV can be added and that cells in Stage IV are typically incubated for 6-9 days.

Page 29: paragraph 87

This describes Stage V. At Stage V the authors advise to withdraw one (or more) of the mitogenic factors. It states that, in addition, many other factors, among them BDNF, NT4, GDNF, dbcAMP, and all trans RA, may be added.

In summary, none of this text, separately or together, suggests the specific claimed protocol. How does the Examiner apply this text to support the rejection? The Examiner addresses the Applicants' arguments

related to sequentially adding growth factors starting on page 7 of the Office Action where, as discussed above, these various textual citations are submitted. On page 8 of the Office Action, the Examiner emphasizes certain text as follows: "Studer also teaches that expanded ES cells from Stage IV are induced to neuronal differentiation in the culture medium in the presence of BDNF and in the absence of SHH and FGF8 for 4-10 days" (Emphasis in the original). Here the Examiner cites, in particular, page 26, paragraph 78. The protocol in page 26, paragraph 78, where BDNF is used in the absence of SHH and FGF8, is in the context of a procedure for generating GABA-ergic neurons. Nowhere in the process is FGF8 or SHH. This procedure involves only using bFGF in Stage IV and, specifically, instructs not to use SHH and FGF8 in Stage IV. So, the only other factor involved in this modification is BDNF, where the reference instructs using, at Stage V, cAMP and BDNF, or cAMP and NT4. Therefore, this protocol for generating GABA-ergic neurons is far from the claimed invention as this particular modification does not involve using two of the claimed factors.

In reference to the underlined section, the Examiner also cites page 29, paragraph 87. This paragraph is focused on dopaminergic neurons. With dopaminergic neurons, the bFGF, SHH, and FGF8 are indeed involved, but bFGF is never used prior to the introduction of FGF8 and SHH. Moreover, the mitogen to be used in Stage IV or V to generate dopaminergic neurons, is chosen from among 16 possible factors. Accordingly, BDNF is only one possibility for Stage IV and/or Stage V. In any event, here the bFGF in this paragraph does not, in fact, teach separately using bFGF separately from FGF8 and SHH, much less using it prior to these two factors.

Further rationale for maintaining the rejection is found in the paragraph spanning pages 8-9. Here, the Examiner provides rationale and "evidence" that "Both Studer and Lee do teach the use of different growth factors separately..." The Examiner states that, for example, Lee and Studer teach that bFGF can be used to expand ES cells. This, in fact, is not correct. The Applicants have reviewed Studer and Lee and have not seen that ES cells are expanded using bFGF. The bFGF is used in an expansion stage, but

not for ES cells. It is used in an expansion stage for the CNS precursors. These CNS precursors are produced in Stage III without any bFGF. And the references do not suggest adding it at this stage.

Moreover, in none of the modifications to produce the specific types of neurons where SHH and FGF8 are used, is bFGF ever used prior to the exposure of cells to those two factors.

Also, on page 8, the Examiner indicates that "In addition to culture medium in the presence of bFGF and in the absence of FGF8 and SHH can increase astrocyte generation." This is also an incorrect statement. For astrocytes, Stage IV indeed contains all of bFGF, SHH, and FGF8. See page 5:17. There is, indeed, an extra step following this Stage IV step where the cells are removed and incubated alone with bFGF, EGF, or PDGF. But, again, the bFGF is either introduced with or after SHH and FGF8.

The Examiner further states that the "culture medium in the presence of BDNF and in the absence of FGF8 and SHH can induce neural differentiation." Applicants point out, however, that this scenario is very different from the claimed scenario. What the Examiner refers to is the modification wherein GABA-ergic neurons are produced. In that modification, SHH and FGF8 are not used. For inducing those neurons, there is only bFGF in Stage IV and cAMP plus BDNF or cAMP plus NT4 at Stage V.

Accordingly, if one examines each of the different modification procedures, it is apparent that each one requires specific sequences and factors. Each modification of the basic five-step protocol is designed with specific parameters, specific sequences, and specific factors for generating specific types of neurons. Each one is a discrete and directed protocol for making a specific cell type. One will not find the claimed protocol in any of these modified protocols; nor is there any reason to modify any of these discrete protocols to arrive at the Applicants' specific claimed protocol. At least, the references do not explicitly or inherently suggest doing this because they have directed the reader about how specifically to modify the protocol for arriving at each of the desired cell types.

In the following section, Applicants set forth ALL of the various modifications in both Studer and Lee.

Five-Step Method and Modification From the Lee Reference

Lee is directed to the production of dopaminergic neurons. Lee does find that the disclosed procedure for producing dopaminergic neurons also produces some serotonergic neurons. But, the reference is limited to methods designed to produce dopaminergic neurons. Applicants have reviewed the teachings of this reference for all permutations and modifications of the methods. These are presented in schematic form in the attached Appendix A. The diagrams are numbered to correspond to the numbers below. Applicants briefly review each of those permutations here.

(1) The Abstract -- The five-step procedure is disclosed for the fourth step only. The text suggests using at least one neurogenic agent, for example, bFGF, SHH, and FGF8.

(2) For Figure 1, which shows the method for making dopaminergic neurons, Stage IV shows incubation with bFGF and laminin.

(3) For Figure 4, this shows that, for making dopaminergic neurons, the optimum procedure at Stage IV is a combination of SHH and FGF8 and indicates that this is detrimental for Stage V.

(4) Paragraph [0016] discloses neurologic agents for proliferation (i.e., Stage IV) and gives as examples bFGF and EGF. This text also suggests other neurologic agents for specific types of neurons and, preferably, SHH, FGF8, or both at Stage IV.

(5) Paragraph [0040] discusses neurogenic agents at Stage IV and provides, as examples, bFGF, SHH, and FGF8. It also states that a combination of SHH and FGF8 at this stage enhances dopaminergic neurons.

(6) Paragraph [0071] discloses an extensive list of potential neurologic agents that could be useful in the methods.

(7) Paragraph [0111] discusses Stage I (expansion of the initial embryonic stem cells) with the time period being generally 4-8 days and, preferably, 6-7 days.

(8) Paragraph [0114] discusses Stage II (formation of embryoid bodies) disclosed as 4-7 days.

(9) Paragraph [0120] discusses Stage III as selection for nestin-positive cells from the embryoid bodies (i.e., "CNS precursor cells") with a duration of about 6-8 days.

(10) Paragraph [0122] is directed to expansion of the nestin-positive/CNS precursor cells.

(11) Paragraph [0123] shows that Stage IV, wherein the nestin-positive cells are expanded, has a duration of about 6-7 days.

(12) Paragraph [0124] discusses Stage IV CNS proliferation medium and that the medium may be supplemented with neurologic agents to encourage the differentiation into neurons; these agents are, preferably, bFGF or EGF.

(13) Paragraph [0125] teaches that Stage IV may be supplemented with neurologic agents to increase the efficiency of the generation of dopaminergic neurons. These can be bFGF or EGF plus SHH and FGF8.

(14) Paragraph [0126] discusses experiments where SHH and FGF8 are added at Stage I or Stage II. It indicates that this is less effective than when these are added at Stage IV [for forming dopaminergic neurons].

(15) Paragraph [0127] discusses Stage V in which the expanded CNS precursors are differentiated.

(16) Paragraphs [0188] to [0204] in the Example section disclose that the invention is directed to methods for making dopaminergic neurons from embryonic stem cells rather than GABA-ergic or glutaminergic neurons. The five steps are, in duration, respectively: 6 days, 5 days, 6 days, 6-7 days, and

5-6 days. Stage IV involves culture with bFGF, FGF8, and SHH. At Stage V, the bFGF is removed. The text indicates that FGF8 and SHH are detrimental at Stage V.

(17) Figure 6 shows that, in the procedure of generating dopaminergic neurons, there was also some generation of serotonergic neurons. SHH works just as well as SHH and FGF8 in Stage IV. Accordingly, FGF8 could be deleted for making serotonergic neurons.

It is evident, when considering all of the teachings of this reference, individually or combined, that there is no teaching or suggestion to use the claimed protocol. In the disclosed procedure, the bFGF, FGF8, and SHH at Stage IV are all administered together for 6-7 days. While it is true that there is a suggestion that FGF8 and SHH might be administered at Stages I and II (bringing it to a total of 18 days for FGF8 and SHH), this still does not teach or suggest the claimed combination of factors, sequence, and time.

Furthermore, combining this reference with Studer does not overcome these deficiencies. Studer, like Lee, does not teach or suggest the claimed protocol.

<u>Five-Step Method and Modification From the Studer Reference</u>

Studer is directed to the production not only of dopaminergic neurons, but also of other neural-related cells. Applicants have reviewed the teachings of this reference for all permutations and modifications of the methods. These are presented in schematic form in the attached Appendix B. The diagrams are numbered to correspond to the numbers below. Applicants briefly review each of those permutations here.

(1) 3:11 – The text provides an overview basically the same as the Lee reference. It does not refer to neurologic agents, but instead refers to growth factors and/or mitogens. At Stage IV, the nestin-positive (CNS precursors) cells are exposed to a growth factor and/or mitogen and then, at Stage V, as in Lee, the mitogen is withdrawn.

(2) 4:14 – The embryoid bodies are cultured for 9-16 days to produce nestin-positive cells. This is different from Lee, which teaches around 6 days. This is Stage III. The text teaches that after this 9-16 days, the mitogen is added to the nestin-positive cells, the mitogen being SHH, FGF8, and laminin. As with Lee, the mitogen is then withdrawn at Stage V.

(3) 4:15 – This text discusses dopaminergic neurons. The mitogen is bFGF. It is preferred that SHH and FGF8 are used as well. This is Stage IV and all are added together. The text also provides a long list (16) of other mitogens/factors and suggests that one or more of these may also be added at Stage IV. One of these is BDNF.

(4) 5:16 – This describes the method for making serotonergic neurons. All that is disclosed is that bFGF is added at Stage IV.

(5) 5:17 – This text discusses modifications for making astrocytes. Here, after Stage IV is completed with bFGF, SHH, and FGF8, there is an extra stage where the Stage IV embryoid bodies are removed and incubated alone with bFGF, EGF, or PDGF prior to Stage V.

(6) 5:18 – This text describes modifications for oligodendrocytes. This also involves the typical Stage IV incubation of bFGF, SHH, and FGF8. But there is an additional Stage IV step. The additional Stage IV step is incubation with bFGF plus EGF or bFGF plus CNTF.

(7) 5:19 – This is for GABA-ergic neurons. Here, bFGF is added at Stage IV and SHH and FGF8 are deliberately not used. Then BDNF and cAMP or NT4 and cAMP are used at Stage V.

(8) 8:31 – This discusses Figure 5 and shows five distinct steps involved with creating dopaminergic neurons. The factors, again, are shown only with Stage IV. All the following text refers to exemplary material.

(9) 17-59 – The Background refers to a Lee protocol (*Nature Biotechnology*, 18:675-679 (2000)) for creating dopaminergic and serotonergic neurons (Appendix C).

(10) 24:71 – The text states that dopaminergic differentiation is done as described by Lee, with a crucial modification being 9-16 days at Stage III instead of 6 days. This reference discusses only the creation of dopaminergic and serotonergic neurons from mouse ES cells *in vitro*.

(11) 24:74 – This pertains to Example 2 and discusses Figure 5, showing generally five steps for creating dopaminergic neurons. This has been discussed above.

(12) 25:76 – The text again states that efficient differentiation of dopaminergic neurons requires FGF8 and SHH in Stage IV in addition to the bFGF.

(13) 25:77 – To promote dopaminergic differentiation at Stage IV and/or V, factors that affect dopaminergic neuron induction and survival can be added. A list of 16 different factors, including BDNF, are listed.

(14) 26:78 – This text discusses a modification to generate astrocytes. Here, as discussed above, there is an extra step 4, comprising bFGF plus EGF or an extra step for comprising bFGF plus CNTF. The text also discusses a modification to produce oligodendrocytes, specifically, an extra step after Stage IV with bFGF, BFG, and PDGF. For both astrocytes and oligodendrocytes, presumably Stage IV is the typical exposure to bFGF, SHH, and FGF8.

(15) 28:85 – This discusses Stage IV and modifications for different cell types. This repeats what is directly above for (14). It indicates that bFGF is typical and SHH and FGF8 are used for dopaminergic neurons, EGF and CNTF are used for astrocytes, SHH is used for oligodendrocytes, and Stage IV replating is used for making glia. This is consistent with and explained in more detail in (14) above.

(16) 29:86 – This text indicates other factors that can be used at Stage IV to inhibit dopaminergic and serotonergic neurons and suggests bone morphogenic protein (BMP).

(17) 29-87 – This discusses Stage V and indicates that typically there are no mitogens. It also states that one may add one or more of the 16 factors to promote dopaminergic differentiation and survival. These sixteen factors are the same discussed above in (13) 25:77.

Studer does not discuss duration, except for Stage III, which is 9-16 days. However, since the generation of dopaminergic neurons and serotonergic neurons are derived from Lee, at least the duration for the formation of these neurons may be available. Accordingly, Applicants reviewed the reference for this information. On page 676, it indicates that for dopaminergic neurons, that neurons were differentiated for 6 days and 13 days. In the experimental protocol on page 768, it is indicated that embryoid bodies would form for 5 days; selection of nestin-positive cells, for 6-10 days, with expansion of nestin-positive cells for 6 days (Stage IV); and differentiation for 6-15 days.

Accordingly, this reference explicitly presents modifications of the dopaminergic/serotonergic differentiation protocol of Lee to produce not only dopaminergic/serotonergic neurons but also other cell types. But none of these modifications provides the particular claimed combination of factors, sequence, and duration.

The schematics in Appendix A and B show visually what the various modifications are. As is evident, none of the text alone, or in combination, in either reference or in both references combined, can be shown to suggest the claimed method of subjecting a stem cell to bFGF first for at least 7 days, then culturing the cells produced with FGF8 and SHH for at least 7 days, then culturing the cells produced in that last step with BDNF for at least 7 days, and then co-culturing the cells produced in that last step with astrocytes. If the Examiner disagrees, she is respectfully requested to point out text that would provide this specific and particular combination of factors, sequence, and duration.

Applicants have adequately addressed the rejection of the claim over these references and overcome the rejection. Reconsideration and withdrawal of the rejection is, therefore, respectfully requested.

B. Rejection Under 35 U.S.C. § 103: Studer/Lee/Song

On page 11 of the Office Action, claims 1-11 and 13 are rejected on the grounds that they are unpatentable over WO 02/086073 and U.S. 2003/0211605 and further in view of Song et al. (*Methods in Molecular Biology*, 198:79-88 (2002)). The Examiner maintains the rejection for reasons of record. Applicants respectfully traverse the rejection.

Song is used to address claims directed to multipotent adult progenitor cells in bone marrow per claims 7-10. The basis for the rejection is that these are stem cells and, therefore, it is obvious to differentiate them into neurons. Applicants respectfully disagree.

Song does not compensate for the deficiencies of WO 02/086073 and U.S. 2003/0211605. These references are deficient against the currently amended claims for reasons give in detail above. Song does not compensate for these deficiencies. Song is not directed, as the Examiner recognizes, to any differentiation protocol at all, but merely as teaching stem cells. Accordingly, the maintenance of the rejection over this combination of references is improper and should be withdrawn.

Applicants have adequately addressed the rejection of the claim over these references and overcome the rejection. Reconsideration and withdrawal of the rejection is, therefore, respectfully requested.

C. Rejection of the Claims Under 35 U.S.C. § 112, First Paragraph

On page 12 of the Office Action, claims 7 and 8 have been rejected on the grounds that they do not comply with the written description requirement, indicating "this is a <u>new matter</u> rejection." (Emphasis is in the original.) Specifically, the Examiner objects to the phrase "cells that are not embryonic stem cells, embryonic germ cells, or germ cells and can differentiate into at least one cell type of each of the

endodermal, ectodermal, and mesodermal embryonic lineages." The Examiner indicates that this was not disclosed in the specification and, thus, introduces new matter. Applicants respectfully traverse the rejection.

First, Applicants point out that there is precedent in the USPTO that contradicts the Examiner's determination that this is a new matter rejection. Applicants direct the Examiner to Applicants' related applications 11/238,234 and 10/467,963. The Examiner will note than in neither prosecution has the Examiner rejected the claims based on inadequate written description. Applicants incorporated the parent application (now U.S. Patent 7,015,037) into 11/238,234 and 10/467,963 as well as into the present application. See "Related Applications/Patents and Incorporation by Reference." In an amendment in 11/238,234, Applicants pointed out support, for example, in the Summary of the Invention, paragraph spanning pages 8 and 9 and the first full paragraph of page 9. These pages are attached for the Examiner's convenience. Accordingly, Applicants believe, and there is USPTO precedent, that the phrase to which the Examiner objects as new matter is not, in fact, new matter and is adequately described.

Applicants have adequately addressed the rejection of the claim over these references and overcome the rejection. Reconsideration and withdrawal of the rejection is, therefore, respectfully requested.

IV. Conclusion

None of the text cited by the Examiner explains the obviousness of making the claimed changes. The directions are quite specific regarding each of the neurons with respect to order and presence of the various factors. Thus, the prior art methods are quite specific. At the very least, in the one method where all four factors are present, SHH, FGF8, and bFGF are simultaneously applied and/or SHH and FGF8 are applied prior to bFGF.

The Declaration of Dr. Verfaillie explains why it would not have been reasonable to expect that one would obtain the same results incubating the starting cell with the factors sequentially versus simultaneously. The Examiner has dismissed the Declaration, saying that Dr. Verfaillie did not present side-by-side comparisons. But this is not required to rebut the obviousness rejection. What Dr. Verfaillie opines about, and what obviousness is based on, is what the person or ordinary skill would have expected. Accordingly, the Examiner has erroneously dismissed the Declaration.

In view of the Applicants' argument, Applicants believe that the pending claims are in condition for allowance. Early notification to that effect is respectfully requested. If it is believed that a further interview will expedite prosecution, the Examiner is invited to contact Applicants' attorney Anne Brown at 216-566-8921.

Applicants believe that fees for a two-month extension of time are due with this filing. Such fee is being simultaneously paid via electronic funds transfer with this submission. The Commissioner is hereby authorized to charge any additional fees required or to credit any overpayment to Deposit Account 20-0809. The applicant(s) hereby authorizes the Commissioner under 37 C.F.R. §1.136(a)(3) to treat any paper that is filed in this application which requires an extension of time as incorporating a request for such an extension.

Respectfully submitted,

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March 29, 2010

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Appendix A

Lee

(1) $\longrightarrow \quad \longrightarrow \quad \longrightarrow \quad \xrightarrow[\substack{\text{bFGF}\\ \text{SHH}\\ \text{FGF8}}]{} \quad \longrightarrow$

(2) $\longrightarrow \quad \longrightarrow \quad \longrightarrow \quad \xrightarrow[\substack{\text{bFGF}\\ \text{laminin}}]{} \quad \longrightarrow$

(3) $\longrightarrow \quad \longrightarrow \quad \longrightarrow \quad \xrightarrow[\substack{\text{SHH}\\ \text{FGF8}}]{} \quad \longrightarrow$

(6) $\longrightarrow \quad \longrightarrow \quad \longrightarrow \quad \xrightarrow[\substack{\text{bFGF}\\ \text{SHH}\\ \text{FGF8}}]{} \quad \longrightarrow$

(8) $\xrightarrow[\substack{4\text{-}8\\ 6\text{-}7}]{} \quad \longrightarrow \quad \longrightarrow \quad \longrightarrow \quad \longrightarrow$

(9) $\longrightarrow \quad \xrightarrow[4\text{-}7]{} \quad \longrightarrow \quad \longrightarrow \quad \longrightarrow$

(10) $\longrightarrow \quad \longrightarrow \quad \xrightarrow[6\text{-}8]{} \quad \longrightarrow \quad \longrightarrow$

(12) $\longrightarrow \quad \longrightarrow \quad \longrightarrow \quad \xrightarrow[6\text{-}7]{} \quad \longrightarrow$

(14) $\longrightarrow \quad \longrightarrow \quad \longrightarrow \quad \xrightarrow[\substack{\text{bFGF}\\ \text{or}\\ \text{EGF}\\ +\\ \text{SHH} + \text{FGF8}}]{} \quad \longrightarrow$

(15) $\xrightarrow[\substack{\text{SHH}\\ \text{FGF8}}]{} \quad \xrightarrow[\substack{\text{SHH}\\ \text{FGF8}}]{} \quad \longrightarrow \quad \xrightarrow[\substack{\text{bFGF}\\ \text{or}\\ \text{EGF}\\ (\text{SHH ?}\\ \text{FGF8 ?})}]{} \quad \longrightarrow$

(17) $\xrightarrow{6} \quad \xrightarrow{5} \quad \xrightarrow{6} \quad \xrightarrow[\substack{\text{bFGF}\\ \text{SHH}\\ \text{FGF8}}]{6\text{-}7} \quad \xrightarrow{5\text{-}6}$

Appendix B

Studer

(2) $\longrightarrow \longrightarrow \xrightarrow[9\text{-}16]{} \xrightarrow[\text{SHH, FGF8}]{} \longrightarrow$

(3) $\longrightarrow \longrightarrow \longrightarrow \xrightarrow[\text{bFGF, SHH, FGF8, [list of 16]}]{} \longrightarrow$

(4) $\longrightarrow \longrightarrow \longrightarrow \xrightarrow[\text{bFGF}]{} \longrightarrow$

(5) $\longrightarrow \longrightarrow \longrightarrow \xrightarrow[\text{bFGF, SHH, FGF8}]{} \longrightarrow$

↑ extra step bFGF, EGF, or PDGF

(6) $\longrightarrow \longrightarrow \longrightarrow \xrightarrow[\text{bFGF, SHH, FGF8}]{} \longrightarrow$

↑ extra step bGF + EGF or bFGF + CNTF

(7) $\longrightarrow \longrightarrow \longrightarrow \xrightarrow[\text{bFGF NO SHH or FGF8}]{} \xrightarrow[\text{BDNF + AMP or NT4 + AMP}]{}$

(10) $\xrightarrow[?]{} \xrightarrow[4]{} \xrightarrow[9\text{-}16]{} \xrightarrow[6]{} \xrightarrow[6\text{-}15]{}$

(12) $\longrightarrow \longrightarrow \longrightarrow \xrightarrow[\text{FGF8, bFGF, SHH}]{} \longrightarrow$

(13) $\longrightarrow \longrightarrow \longrightarrow \xrightarrow[\text{bFGF, SHH, FGF8, [list of 16]}]{} \xrightarrow[\text{[list of 16]}]{}$

(14) $\longrightarrow \longrightarrow \longrightarrow \longrightarrow \longrightarrow$

↑ extra step bFGF + EGF or bFGF + CNTF

$\longrightarrow \longrightarrow \longrightarrow \longrightarrow \longrightarrow$

↑ extra step bFGF EGF PDGF

$\longrightarrow \longrightarrow \longrightarrow \xrightarrow[\text{bFGF NO SHH FGF8}]{} \xrightarrow[\text{BDNF + cAMP or NT4 + cAMP}]{}$

(17) $\longrightarrow \longrightarrow \longrightarrow \longrightarrow \xrightarrow[\text{[list of 16]}]{}$

Appendix C

Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells

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Received 15 April, 2000; accepted 24 April, 2000

Embryonic stem (ES) cells are clonal cell lines derived from the inner cell mass of the developing blastocyst that can proliferate extensively in vitro and are capable of adopting all the cell fates in a developing embryo. Clinical interest in the use of ES cells has been stimulated by studies showing that isolated human cells with ES properties from the inner cell mass[1,2] or developing germ cells[3] can provide a source of somatic precursors. Previous studies have defined in vitro conditions for promoting the development of specific somatic fates, specifically, hematopoietic, mesodermal, and neurectodermal[4-7]. In this study, we present a method for obtaining dopaminergic (DA) and serotonergic neurons in high yield from mouse ES cells in vitro. Furthermore, we demonstrate that the ES cells can be obtained in unlimited numbers and that these neuron types are generated efficiently. We generated CNS progenitor populations from ES cells, expanded these cells and promoted their differentation into dopaminergic and serotonergic neurons in the presence of mitogen and specific signaling molecules. The differentation and maturation of neuronal cells was completed after mitogen withdrawal from the growth medium. This experimental system provides a powerful tool for analyzing the molecular mechanisms controlling the functions of these neurons in vitro and in vivo, and potentially for understanding and treating neurodegenerative and psychiatric diseases.

To understand the developmental transitions of stem cells and fulfill their clinical promise, it must be possible to control the differentiation of ES cells into specific cell fates. Neurons, astrocytes, and oligodendrocytes have been derived from ES cells[7-10]. The neurotransmitters, dopamine and serotonin, have important functions in the etiology and treatment of neurodegenerative[11] and psychiatric diseases[12-15]. The neurons that synthesize these transmitters are generated by similar developmental signals around the boundary between the midbrain and the hindbrain.

To assess the developmental progression of ES cells in vitro, we examined the appearance of CNS- and midbrain-specific gene products in a modification of our previous work where CNS stem cells were derived from ES cells[10]. The differentiation involves several steps: the generation of embryoid bodies (EBs; stage 2), the use of a defined medium to select for CNS stem cells (stage 3), the proliferation of CNS stem cells in the presence of mitogen, basic fibroblast growth factor (bFGF; stage 4), and the differentiation of the stem cells by removal of the mitogen (stage 5; Fig. 1A).

The successful conversion of ES cells into midbrain dopaminergic (DA) neurons in vitro should be dependent on the induction of the same genes that are expressed in CNS stem cells and neurons in vivo. The *OTX* homeobox genes (*OTX1* and *OTX2*) are widely expressed at early stages of neurectoderm differentiation[16]. *OTX2* is expressed throughout the epiblast and subsequently restricted to anterior neurectoderm, where it is required for development of the forebrain and midbrain. The homolog *OTX1* is first expressed in the neurectoderm in the dorsal telencephalon, and interactions between these two *OTX* genes are thought to specify the development of the midbrain and hindbrain[17]. *OTX2* is expressed in the undifferentiated (stage 1) ES cells and present at lower levels at stage 2 and 3 of differentiation. *OTX1* is not expressed at high levels until stage 3 (Fig. 1B).

Several genes (*Pax2, Pax 5, Wnt1, En1, Nurr1*) have been identified that control differentiation of dopaminergic and serotonergic neurons in the midbrain and hindbrain[18,19]. PCR analysis detected expression of these genes at stages 3 and 4 of ES cell differentiation. After stage 4, the bFGF-expanded cells are morphologically uniform and express nestin, an intermediate filament protein characteristic of CNS stem cells (Fig. 2A)[20,21]. These results suggest that ES cells show a progressive restriction to mesencephalic and metencephalic CNS stem cells.

Identification of neurons synthesizing neurotransmitters expected for midbrain and hindbrain fates would lend support to this conclusion. Tyrosine hydroxylase (TH) is the rate-limiting enzyme for the biosynthesis of dopamine and a marker of ventral midbrain neurons. No TH expression was detected at stages 1–4 of ES cell culture (data not shown). However, the stage 4 cells may be primed for induction of TH, but still require time and appropriate conditions to express differentiated neuronal features. Differentiation (stage 5) was promoted by using conditions known to promote neuronal differentiation from the proliferating precursor state[22-24]. TuJ1, an antibody directed against the neuron-specific β-III tubulin bound many cells with a clear neuronal morphology (Fig 2B). Of the total cell population, 71.9 ± 6.9% were TUJ1$^+$, and 6.9% ± 1.5% of the TUJ1$^+$ cells were also TH$^+$ (these numbers are the average ± s.e.m. of three independent experiments, n > 40 microscopic fields, for a total containing 15.7×10^3 neurons). The

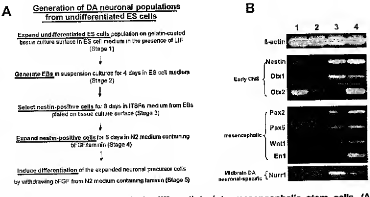

Figure 1. ES cells progressively differentiate into mesencephalic stem cells. (A) General scheme of ES cell culture. (B) Differentiated ES cells express genes characteristic of midbrain fates. Semi-quantitative RT-PCR analysis of the early CNS, mesencephalic, and midbrain-specific regulatory genes at different stages of ES cell culture. Numbers at the top of the panel designate stages of culture defined in (A).

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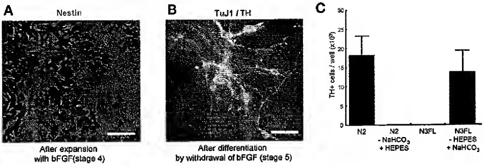

Figure 2. Mesencephalic precursors differentiate into TH⁺ neurons. (A) ES cell progeny at stage 4 express nestin, an intermediate filament protein expressed in CNS stem cells but not in neurons and glia (scale bar = 20 μm). (B) ES cell progeny at stage 5 differentiate into TH⁺ neurons. Removal of the mitogen initiates differentiation of the nestin-positive cells into neurons recognized by the antibody TuJ1 that binds to a neuron-specific tubulin. A subset of the neurons expresses the enzyme tyrosine hydroxylase (TH), which is required for the production of catecholamine neurotransmitters (scale bar = 20 μm). (C) The effect of HEPES buffer on differentiation of TH⁺ cells. N3FL is a medium used for ES culture[10].

neuronal differentiation is as efficient as has been previously achieved from primary CNS stem cells[22,25]. This result suggests that the ES-derived nestin-positive population is dominated by CNS stem cells and differentiates into midbrain neurons.

We found that the method of EB formation and the composition of growth medium exert a clear effect on the yield of TH⁺ neurons. The significant percentage of TH⁺ neurons among the differentiated ES cell progeny contrasts with previous studies in which no appreciable numbers of TH⁺ cells were observed[10]. The current procedure was achieved by two modifications that optimized neuronal differentiation and TH expression. In this work ES cells were first dissociated into single cells to obtain a more uniform procedure for EB formation. As a result of this modification the yield of nestin-positive cells, as determined after stage 3, was increased by 3.5-fold. The second modification was to use medium without HEPES during stages 4 and 5 of culture. HEPES inhibits differentiation of TH⁺ neurons (Fig. 2C). This modification of the culture conditions leads to the efficient production of TH⁺ neurons.

Sonic hedgehog (SHH), FGF8, and ascorbic acid (AA) increased the yield of ES-derived TH⁺ neurons. FGF8 and SHH have been previously shown to promote ventral midbrain fates in neural plate explant[25], therefore we hypothesized that the addition of these factors at specific stages during an in vitro ES cell differentation might also increase the proportion of DA and serotonergic neurons in our culture system. As shown in Figure 3A, the SHH receptors (smoothened, Smo, and patched, Ptc), a downstream transcription factor (Gli1), the FGF8 receptor (FGFR3), and the ligands (SHH and FGF8) are expressed during ES cell differentiation. Combined treatment with SHH/FGF8 during stage 4 leads to a greater than twofold increase in the number of TH⁺ cells (Fig. 3B; 15.4 ± 2.4% of the TuJ1⁺ neurons; value of three independent experiments, $n > 40$ fields containing 15.7×10^3 neurons, $P < 0.01$). Application of SHH/FGF8 at earlier (stage 2 or 3) or later (stage 5) stages had no effect on the number of TH⁺ neurons (data not shown). When applied as single factors at stage 4, SHH and FGF8 were both significantly less effective than when added in combination.

The cAMP analog, dibutyryl cAMP, and AA have been previously implicated in promoting DA neuron yield from primary CNS cultures[27,28]. Whereas dibutyryl cAMP was ineffective alone or in combination with SHH and FGF8, treatment with AA during stage 5 led to a significant increase in TH⁺ cell yield (Fig. 3B). The percentage of ES-derived TH⁺ neurons after treatment with SHH/FGF8 during stage 4 and AA during stage 5 reached 33.9 ± 5.5% of the neurons ($n > 40$ fields containing 4.2×10^3 neurons) which corresponds to more than 20% of the total cell population. Importantly, we found that the population of TH⁺ cells was maintained when stage 4 cells treated with SHH and FGF8 were passaged before differentiation.

To further characterize the ES-derived TH⁺-cell population we performed double immunohistochemistry for TH and (1) dopamine-β-hydroxylase (DBH), a marker of noradrenergic neurons (2) γ-aminobutyric acid (GABA), a marker for inhibitory neurons, and (3) serotonin, a transmitter found in many ventral hindbrain neurons. No coexpression of TH with any of these markers was observed, suggesting that the TH⁺ cells are DA neurons (data not shown).

The production of dopamine is a definitive measure of the identity of a dopaminergic neuron. Reverse-phase high-performance liquid chromatography (RP-HPLC) was used to measure dopamine release directly (Fig. 3C). The dopamine peak can be readily separated and the sensitivity of the procedure repeatedly confirmed with control samples. Neurons were differentiated in stage 5 for six days, and medium conditioned for 48 h was tested. Consistent with the increase in the number of TH⁺ neurons, the dopamine level was increased more than twofold in cultures treated with SHH/FGF8/AA ($n = 3$, $P < 0.05$). Elevated potassium was used to determine if dopamine could be released by depolarization. When Hank's balanced salt solution (HBSS) was applied to the cells for 15 min, 165.7 ± 23.4 pg ml^{-1} dopamine was detected. SHH/FGF8/AA did not change dopamine levels. However, dopamine levels were elevated when the neurons were depolarized with elevated potassium (56 mM), and the medium collected after 15 min (416.6 ± 7.2 pg ml^{-1} in the controls and 918.4 ± 123.2 pg ml^{-1} of dopamine in the SHH/FGF8/AA-treated cultures). These data demonstrate that ES-derived TH⁺ cells secrete significant levels of dopamine when depolarized.

Electrophysiology was used to determine if ES-derived neurons were functionally active. When current was injected into individual patch-clamped neurons, sustained trains of action potentials, characteristic of mature neurons, were routinely observed ($n = 54$; Fig. 4A). The cells also responded to direct application of the neurotransmitters GABA and glutamate (Fig. 4B, C). After 13 days of differentiation at stage 5, the neurons showed spontaneous activity that was almost completely blocked by tetrodotoxin (TTX), an inhibitor of action potentials (Fig. 4D, E). Biocytin labeling demonstrated that neurons exhibiting TTX-sensitive spontaneous activity were TH⁺ (Fig. 4F, G). This demonstrates that TH⁺ cells receive synaptic input and suggests that this system can be used to define synaptic control of dopamine release.

In addition to specification of midbrain DA precursors, SHH is also important for the generation of hindbrain serotonergic neurons[26]. As shown in Figure 5, in untreated cultures 0.8 ± 0.1% ($n = 56$, fields containing 6.2×10^3 neurons) of all TuJ1⁺ neurons were serotonin-positive. However, addition of SHH/FGF8 during stage 4 increased the serotonergic population by 14-fold (11.0 ± 0.5%, $n = 56$ fields containing 6.2×10^3 neurons, $P < 0.01$ of all TuJ1⁺ cells). Serotonin and TH were not coexpressed. Interestingly, application of SHH alone promoted serotonergic fates to an extent similar to the combined treatment (Fig. 5B). These results are in agreement with

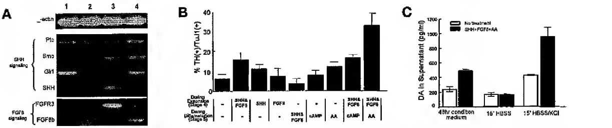

Figure 3. The effect of external signals on the yield of neurons that are TH⁺ and secrete dopamine. (A) Expression of genes in the SHH and FGF8 signal pathways. Semiquantitative RT-PCR analysis at different stages of cell culture. Numbers at top of the panel designate stages of the culture; see Figure 1A. (B) The yield of TH⁺ neurons is expressed as a percentage of TuJ1⁺ neurons. SHH (500 ng ml⁻¹), FGF8 (100 ng ml⁻¹), cAMP (1 mM), and AA (200 μM) were added at different stages of ES cell development, as shown. (C) The effect of treatment with SHH, FGF8, and AA on maturation of DA neurons as measured by dopamine release. The RP-HPLC determination of dopamine concentration is shown in medium (N2) conditioned for 48 h (left), in HBSS conditioned for 15 min (center), and in HBSS + 56 mM KCl conditioned for 15 min (right).

findings in neural plate explant cultures suggesting that SHH can act alone to specify the precursors for serotonergic neurons [26]. The efficient induction of serotonergic neurons by SHH suggests that these differentiation conditions support hindbrain serotonergic fates.

It has been shown that ES cells can differentiate into neurons and glia in vitro[7,10] and in vivo[8,9,29]. However, no systematic derivation of midbrain and hindbrain neurons from ES cells has been reported. Here we found that catecholaminergic and serotonergic neurons can be efficiently derived from ES cells. HEPES buffer addition during stage 4 and stage 5 was incompatible with DA differentiation of ES cells. At present the mechanism of this inhibitory effect is not known. SHH, FGF8, and AA also enhance the differentiation to dopaminergic and serotonergic fates. Less than 5% of the neurons express TH when mesencephalic cells are directly differentiated without expansion in the precursor state[30]. When E12 rat mesencephalic precursors were first expanded in bFGF, 25% of the neurons were TH⁺ (ref. 24). This ES cell system gives access to different stages of development leading to >30% of TH⁺ neurons, the highest yield ever obtained either in vitro or in vivo.

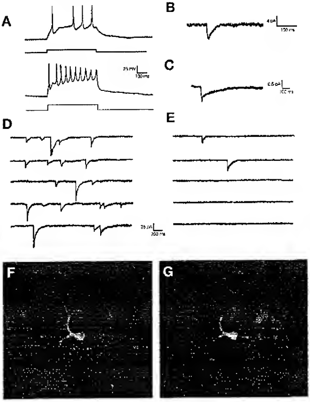

Figure 4. Synaptic properties of ES-derived TH⁺ neurons. (A) Action potential spiking behavior. Following 13 days of differentiation (stage 5), depolarization causes the cell to fire an initial action potential followed by a few others at low frequency. With increasing amounts of depolarization the neuron will fire a train of action potentials at a higher frequency. This behavior was common in the cultures and is characteristic of mature neurons. (B) GABA application to the dendrites of a neuron leads to an inward current. (C) Glutamate application to the dendrites of a neuron leads to an inward current. (D) Spontaneous activity in TH⁺ neurons recorded in voltage clamp mode. (E) Tetrodotoxin (TTX) blocks spontaneous activity in TH⁺ neurons. The activity in the same cell as shown in (D) was diminished when action potentials were blocked with 1 μM TTX. Note that the TTX blocks almost all spontaneous activity, indicating that most of the activity is synaptic release of transmitter evoked by spontaneous action potential firing in presynaptic neurons. (F, G) The cell recorded in (D) and (E) is TH⁺. The cell tracer biocytin was introduced through the recording electrode, and confocal images are presented showing the colocalization of TH (F) and biocytin (G) (Scale bars = 20 μm).

The development of cell therapy is a potential advantage of the directed differentiation of ES cells into DA neurons. Although later precursors such as CNS stem cells can also be expanded and differentiated into DA neurons, these have only a limited ability to expand in vitro and to adopt a dopaminergic fate; moreover, these cells do not produce more DA neurons in response to SHH and FGF8 treatment (our unpublished observation). The ES cells, on the other hand, proliferate without limit, respond to signaling molecules, and are easily accessible to genetic manipulation. We anticipate that the efficiency of the system described here can be further improved by generating ES cell lines expressing regulatory genes such as *Nurr1* known to control commitment to DA fate in vivo[31]. We also expect that the purification of a homogeneous midbrain population can be achieved by genetic methods. Although studies in Parkinsonian rodents are needed to further assess the function and safety of ES cell-derived DA neurons in vivo, our data demonstrate that the neurons derived from ES cells produce dopamine, respond to neurotransmitters, and exhibit spontaneous synaptic activity.

The central finding of this study is that midbrain and hindbrain neurons can be generated in unlimited numbers from ES cells. For every 3×10^6 of ES cells, we obtain 21×10^6 neurons and 7×10^6 DA neurons. Numerically, two to three TH⁺ cells are harvested at stage 5 for every undifferentiated ES cell plated at stage 1. This represents the sum total of all parameters of proliferation, cell death, and selective differentiation throughout the five culture stages. There are 3×10^4 DA neurons in the rat substantia nigra, and 10^3 grafted DA cells lead to behavioral recovery in a rat model of Parkinson's disease[25]. Genetic manipulation may now be used to obtain pure populations of the DA neuron suitable for grafting and other experiments relevant to the etiology and therapy of Parkinson's disease.

The dopamine- and serotonin-synthesizing neurons are two ventral fates generated in vivo, anterior and posterior of the isthmic organizer, a source of signaling molecules that control the differentiation

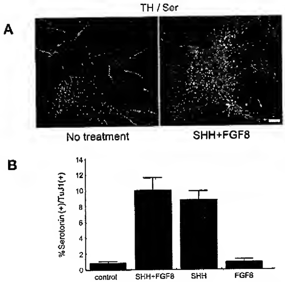

Figure 5. Characterization of serotonin-positive neurons. (A) Immunostaining shows serotonin-positive neurons. Serotonin and TH-positive neurons are shown by immunostaining cultures treated at stage 4 in the absence (left) or presence (right) of SHH (500 ng ml^{-1}) and FGF8 (100 ng ml^{-1}). The yield of serotonergic neurons over the untreated controls in the presence of the growth factors (scale bars = 20 μm). (B) The yield of serotonin-positive neurons (expressed as a percentage of all TuJ1$^+$ neurons) is shown for cells treated with different growth factor combinations at stage 4. In this case, AA was not used at stage 5.

midbrain and hindbrain patterning[32]. Our findings show that under this protocol almost half of the neurons generated can adopt a ventral mid/hindbrain fate. This will facilitate understanding the mechanisms controlling early steps in the differentiation of these neurons that have previously been difficult to access. For example, at present in vivo and explant systems are not well suited to define if SHH and FGF8 act through inductive or selective mechanisms, but clonal analysis in the dissociated ES cells system will answer this question. As we demonstrate, this system is also well suited to analyze mechanisms of neuronal differentiation, function, and survival.

Experimental protocol

Maintenance of undifferentiated ES cells (R1, E14.1, B5—all three lines of ES cells behaved similarly), EB formation, and selection of nestin-positive cells were carried out as described[10] with modifications. Undifferentiated (stage 1) ES cells were grown on gelatin-coated tissue culture plates in the presence of 1,400 U ml^{-1} of leukemia inhibitory factor (LIF; GIBCO/BRL, Grand Island, NY) in ES cell medium consisting of knockout Dulbecco's minimal essential medium (DMEM; GIBCO/BRL) supplemented with 15% FCS, 100 mM MEM nonessential amino acids, 0.55 mM 2-mercaptoethanol, L-glutamine, and antibiotics (all from GIBCO/BRL). To induce EB formation (stage 2), the cells were dissociated into a single-cell suspension by 0.05% trypsin and 0.04% EDTA in PBS and plated onto nonadherent bacterial culture dishes at a density of 2–2.5 × 10^4 cells cm^{-2} in the medium described above. The EBs were formed for four days and then plated onto adhesive tissue culture surface in the ES cell medium. After 24 h of culture, selection of nestin-positive cells (stage 3) was initiated by replacing the ES cell medium by serum-free Insulin/Transferrin/Selenium/Fibronectin (ITSFn) medium[10].

After 6–10 days of selection, cell expansion (stage 4) was initiated. Specifically, the cells were dissociated by 0.05% trypsin/0.04% EDTA, and plated on tissue culture plastic or glass coverslips at a concentration of 1.5–2 × 10^5 cells cm^{-2} in N2 medium modified according to Johe and colleagues[22], and supplemented with 1 μg ml^{-1} of laminin and 10 ng ml^{-1} of bFGF (R&D Systems, Minneapolis, MN) in the presence or absence of murine N-terminal fragment of SHH (500 ng ml^{-1}) and murine FGF8 isoform b (100 ng ml^{-1}; both from R&D Systems). Before cell plating, dishes and coverslips were precoated with polyornithine (15 mg ml^{-1}) and laminin (1 μg ml^{-1}, both from Becton Dickinson Labware, Bedford, MA). Nestin-positive cells were expanded for six days. The medium was changed every two days. Differentiation (stage 5) was induced by removal of bFGF. The differentiation medium consisted of N2 medium supplemented with laminin (1 mg ml^{-1}) in the presence or absence of cAMP (1 μM), AA (200 μM, both from Sigma, St. Louis, MO). The cells were incubated under differentiation conditions for 6–15 days.

Immunocytochemistry. Cells were fixed in 4% paraformaldehyde/0.15% picric acid in PBS. Immunocytochemistry was carried out using standard protocols. Antibodies and dilutions were as follows: TH polyclonal 1:200 (Pel-Freeze, Rogers, AR) or TH monoclonal 1:1000 (Sigma), β-tubulin type III (TuJ1) monoclonal 1:500 (Babco, Richmond, CA), GABA polyclonal 1:1000 (Sigma), DBH polyclonal 1:100 (Protos Biotech Corp., New York, NY), or DBH polyclonal 1:100 (Pharmingen, San Diego, CA), serotonin polyclonal 1:4000 (Sigma), and fluorescently labeled secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA).

RNA extraction and RT-PCR analysis. Total cellular RNA was prepared using RNAeasy total RNA purification kit (Qiagen, Valencia, CA) followed by treatment with RNase-free RQ DNase (Promega Corp., Madison, WI). For cDNA synthesis, random hexamer primers (Gibco/BRL) were used to prime reverse transcriptase (RT) reactions. Using this method it was possible to use the same RT reaction (cDNA) for PCR amplification with different sets of gene-specific primers. The cDNA synthesis was carried out using Moloney murine leukemia virus (M-MLV) Superscript II reverse transcriptase (Gibco/BRL) following the manufacturer's instructions. To analyze relative expression of different mRNAs, the amount of cDNA was normalized based on the signal from ubiquitously expressed actin mRNA. Levels of neural mRNAs at different stages of ES cell culture was compared to that in the undifferentiated ES cells. The PCR was carried out using standard protocols with Taq polymerase (Boehringer-Mannheim, Indianapolis, IN). Cycling parameters were as follows, denaturation at 94° C for 30sec, annealing at 58-61° C for 1min depending on the primer, and elongation at 72° C for 1 min. The number of cycles varied between 25 and 35, depending on the particular mRNA abundance. The number of cycles and the amount of cDNA was chosen in such a way as to select PCR conditions on the linear portion of the reaction curve avoiding "saturation effects" of PCR. The identity of the PCR products was confirmed by sequencing. Primer sequences (forward and reverse), and the length of the amplified products were as follows:

actin (ATGGATGACGATATCGCTG, ATGAGGTAGTCTGTCAGGT, 569); nestin (GGAGTGTCGCTTAGAGGTGC,TCCAGAAAGCCAAGAGAAGC,327); Nurr1 (TGAAGAGAGCGGAGAAGGAGATC, TCTGGAGTTAAGAAATCGGAGCTG, 255); Gli1 (TCCACAGGCATACAGGATCA,TGCAACCTTCTTGCTCACAC,462); Smoothened (Smo) (CTGAGAGTGCCAGAAAAGGG, TCATCATGCTGGAGAACTCG, 370); Patched (Ptc) (CCTCCTTTACGGTGGACAAA, ATCAACTCCTCCTGCCAATG, 272); Wnt1 (ACCTGTTGACGGATTCCAAG,TCATGAGGAAGCGTAGGTCC,462); Otx1 (GCTGTTCGCAAAGACTCGCTAC, ATGGCTCTGGCACTGATACGGATG, 425); Otx2 (CCATGACCTATA CTCAGGCTTCAGG, GAAGCTCCATATCCCTGGGTGGAAAG, 211); Pax2 (CCAAAGTGGTGGACAAGATTGCC, GGGATAGGAAGGACGCTCAAAGAC, 545); Pax5 (CAGATGTAGTCCGCCAAAGGATAG, ATGCCACTGATGGAGTATGAGGAGCC, 451); FGFR3 (ATCCTCGGGAGATGACGAAGAC, GGATGCTGCCAAACTTGTTCTC, 326); Fgf8 (CATGTGAGGGACCAGAGCC, GTAGTTGTTCTCCAGCAGGATC, 312); En1 (TCAAGACTGACTCACAGCAACCCC, CTTTGTCCTGAACCGTGGTGGTAG, 381); Shh (GGAAGATCACAAGAAACTCCGAAC, GGATGCGAGCTTTGGATTCATAG, 354).

Reverse-phase HPLC. Dopamine levels were determined in conditioned medium and in HBSS stabilized with orthophosphoric acid (7.5%)/metabisulfate (0.22 mg ml^{-1}), and stored at -80°C until analysis. Aluminum absorption and HPLC analysis of dopamine have been described[25,33].

Electrophysiology. For electrophysiological recordings, cells grown on 12 mm glass coverslips were transferred to recording medium containing 130 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose (pH 7.35, 325 mOsm). Patch pipettes were filled with 110 mM potassium gluconate, 20 mM KCl, 2 mM Mg-ATP, 10 mM sodium phosphocreatine, 1.0 mM EGTA, 0.3 mM GTP-Tris, and 20 mM HEPES (pH 7.25, 320 mOsm). To label the recorded cells, biocytin (0.2%) was added to the intracellular medium. Recordings, biocytin labeling, and confocal imaging were performed as described[34].

Cell counting and statistics. Uniform random sampling procedure were used for cell counts and quantified using the fractionator technique[12]. Statistical comparisons were made by ANOVA with post hoc Dunnett test

when more than two groups were involved. If data were not normally distributed, a nonparametric test (Mann–Whitney U-test) was used for the comparisons of results. Data were expressed as mean ± s.e.m.

Acknowledgments

The R1 ES cell line was kindly provided by Dr. Heiner Westphal. The second ES cell line used E14.1 was a gift of Dr. Tom Doetschman. We would like to thank Dr. David Panchinsion for providing some of the PCR-primers and for critical discussions of the manuscript.

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Genome-directed primers for selective labeling of bacterial transcripts for DNA microarray analysis

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Received 9 March, 2000; accepted 25 April, 2000

DNA microarrays have the ability to analyze the expression of thousands of the same set of genes under at least two different experimental conditions[1]. However, DNA microarrays require substantial amounts of RNA to generate the probes, especially when bacterial RNA is used for hybridization (50 μg of bacterial total RNA contains approximately 2 μg of mRNA)[2]. We have developed a computer-based algorithm for prediction of the minimal number of primers to specifically anneal to all genes in a given genome. The algorithm predicts, for example, that 37 oligonucleotides should prime all genes in the *Mycobacterium tuberculosis* genome. We tested the usefulness of the genome-directed primers (GDPs) in comparison to random primers for gene expression profiling using DNA microarrays. Both types of primers were used to generate fluorescent-labeled probes and to hybridize to an array of 960 mycobacterial genes. Compared to random-primer probes, the GDP probes were more sensitive and more specific, especially when mammalian RNA samples were spiked with mycobacterial RNA. The GDPs were used for gene expression profiling of mycobacterial cultures grown to early log or stationary growth phases. This approach could be useful for accurate genome-wide expression analysis, especially for in vivo gene expression profiling, as well as directed amplification of sequenced genomes.

We developed a computer algorithm to define the minimal number of oligonucleotides of a given length capable of priming all genes within any genome. Using the genome sequence of *Mycobacterium tuberculosis*, we applied the algorithm, setting the oligonucleotides length at eight or seven bases, and requiring 100% coverage of the 3,924 open reading frames (ORF) in the genome[3] (Fig. 1A). The search was limited to the first 500 bp of each complementary sequence of each ORF to generate long probes for efficient hybridization. The priming efficiency of the mycobacterial genome-directed primers (mtGDPs; 37 primers) was compared to the priming efficiency of seven- or six-nucleotide random primers in a standard reverse transcription reaction. Probes generated from the same mycobacterial RNA (log phase cultures) using mtGDPs or random primers were hybridized simultaneously to the same slides, and the signal intensities of the 960 arrayed genes were calculated and compared[4,5]. There was a high correlation level between the signals of both probes for the whole array of genes ($r = 0.97$). Additionally, signals generated by mtGDPs were significantly higher than random primer-generated signals ($P < 0.05$) (Fig. 1B, C). Signal intensities